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# Curcumin Suppresses AP1 Transcription Factor-dependent Differentiation and Activates Apoptosis in Human Epidermal Keratinocytes*

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**Sivaprakasam Balasubramanian**[‡] **and Richard L. Eckert**[‡§¶‖**1]

*From the Departments of* [‡]*Physiology and Biophysics,* [§]*Dermatology,* [¶]*Biochemistry,* [‖]*Reproductive Biology, and* [**]*Oncology, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106-4970*

**The diet-derived cancer preventive agent, curcumin, inhibits skin cancer cell proliferation and tumor formation. However, its effect on normal human keratinocyte differentiation, proliferation, and apoptosis has not been adequately studied. Involucrin (hINV) is a marker of keratinocyte differentiation and a useful model for the study of chemopreventive agent action. We show that curcumin suppresses the differentiation agent-dependent activation of hINV gene expression and that an AP1 transcription factor DNA binding site in the hINV gene is required for this regulation. A protein kinase C, Ras, MEKK1, MEK3 signaling cascade controls hINV expression by regulating AP1 factor level. Curcumin treatment inhibits the novel protein kinase C-, Ras-, and MEKK1-dependent activation of hINV promoter activity and reduces the differentiation agent-dependent increase in AP1 factor level and DNA binding. This reduction requires proteasome function. In addition, curcumin treatment reduces cell number, which is associated with a reduced cyclin and cdk1 levels. Curcumin treatment also suppresses the Bcl-xL level, leading to reduced mitochondrial membrane potential and increased cleavage of procaspases and poly(ADP-ribose) polymerase. These studies provide important insights regarding the mechanism whereby curcumin acts as a chemopreventive agent in normal human epidermis.**

Curcumin, diferuloylmethane, is a naturally occurring dietary agent, derived from the roots of *Curcuma longa* Linn, which has potent anti-cancer properties (1, 2). Dietary or topical application of curcumin inhibits tumor development in 7,12-dimethylbenz(*a*)anthracene/12-*O*-tetradecanoylphorbol-13-acetate (TPA)[2]-induced mouse skin carcinogenesis (3–9). Administration of 0.2–1% curcumin in the mouse diet is sufficient to achieve this response (7–9). Several studies suggest that curcumin influences AP1 factor expression. Curcumin treatment of HeLa cells results in altered AP1 factor expression and activity (10), and dietary curcumin reduces carcinogen-induced c-*fos* expression in the epidermis of 7,12-dimethylbenz(*a*)anthracene/TPA-treated Swiss albino and CD-1 mice (8, 9, 11). Curcumin also reduces c-*jun* and c-*fos* expression in murine keratinocyte JB6 cells (12) and influences the proliferation and survival of other cell lines derived from surface epithelia, including HaCaT cells (10, 13), MDA-1986 cells (14), oral squamous cell carcinoma cells (15), NCTC-2544 transformed keratinocytes (16), and transformed murine keratinocytes (17).

These studies suggest that curcumin can modulate tumor formation *in vivo*, as well as, the proliferation of epidermis-derived established cell lines. However, few studies have examined the impact of curcumin treatment on the function of normal human keratinocytes (18, 19). In the present report, we examine the effect of curcumin on human keratinocyte differentiation using involucrin as a marker of differentiation. Involucrin is expressed in the suprabasal layers in epidermis (20, 21). Because of its differentiation-dependent pattern of expression and because the mechanisms that regulate involucrin expression are well understood (21), the involucrin gene provides an ideal model for understanding the mechanisms whereby chemopreventive agents regulate skin differentiation. We also examine the impact of curcumin treatment on keratinocyte proliferation and apoptosis. Our results suggest that curcumin treatment inhibits keratinocyte proliferation and TPA-dependent activation of involucrin gene expression and enhances keratinocyte apoptosis. The curcumin-dependent suppression of differentiation is associated with reduced AP1 factor expression and DNA binding. A unique observation of these studies is the finding that curcumin suppresses expression of all AP1 factors, suggesting it produces profound changes in AP1-mediated end responses. Moreover, these findings are particularly interesting, because they contrast the mechanism of action of curcumin from that of other chemopreventive agents (18, 22, 23).

## EXPERIMENTAL PROCEDURES

*Chemicals and Reagents*—Phorbol ester (TPA), $Me_2SO$, and curcumin were obtained from Sigma. TPA and curcumin were prepared in $Me_2SO$ and stored at −70 °C. MG132 was purchased from Biomol. Keratinocyte serum-free medium (KSFM),

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[1] To whom correspondence should be addressed: Dept. of Physiology/Biophysics, Case Western Reserve University School of Medicine, 2109 Adelbert Rd., Cleveland, OH 44106-4970. Tel.: 216-368-5530; Fax: 216-368-5586; E-mail: rle2@po.cwru.edu.

[2] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; KSFM, keratinocyte serum-free medium; hINV, human involucrin; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MEKK, MEK kinase; ERK, extracellular signal-regulated kinase; PKC, protein kinase C; C/EBP, CAAT/enhancer-binding protein; cdk, cyclin-dependent kinase.

trypsin, Hanks' balanced salt solution, and gentamicin were obtained from Invitrogen. The chemiluminescence luciferase assay kit and pGL2-basic plasmid were from Promega (Madison, WI). [$\gamma$-$^{32}$P]ATP was purchased from PerkinElmer Life Sciences. Rabbit polyclonal antibodies for API proteins, including Fra-1 (sc-605X), Fra-2 (sc-171X), c-Fos (sc-7202X), FosB (sc-48X), c-Jun (sc-1694X), JunB (sc-46X), and JunD (sc-74X) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal $\beta$-actin (A5441) was purchased from Sigma. Rabbit polyclonal antibody for anti-human involucrin was produced in our laboratory. Mouse monoclonal anti-caspase 8 (#9746) and rabbit polyclonal antibodies specific for caspase 9 (#9502) and caspase 3 (#9665) were purchased from Cell Signaling (Beverly, MA). Rabbit polyclonal antibodies for cdk6 (sc-7181), Bax (sc-493), and mouse monoclonal antibodies specific for p21/Waf1 (sc-6246) and cyclin B1 (sc-245) were purchased from Santa Cruz Biotechnology. Mouse monoclonal antibodies for anti-cyclin D1 (554180) and anti-poly(ADP-ribose) polymerase (55494), and a rabbit polyclonal antibody for anti-Bcl-x (610211) were from BD Pharmingen. Peroxidase-conjugated secondary antibodies, including donkey anti-rabbit IgG (NA934) and sheep anti-mouse IgG (NA931), were obtained from Amersham Biosciences.

*Tissue Culture, Cell Transfection, and Luciferase Assay*—Primary keratinocytes from human foreskin were cultured as described (24). Sixty percent confluent cultures of normal human keratinocytes, maintained in 35-mm dishes, were used for transfection. Fugene-6 reagent (4 $\mu$l) was added to 96 $\mu$l of KSFM, and the mixture was incubated at 25 °C for 5 min. The mixture was then mixed with 2 $\mu$g of involucrin promoter reporter plasmid or, for co-transfection experiments, with 1 $\mu$g of involucrin reporter plasmid, and 1 $\mu$g of kinase expression plasmid. The mixture was then incubated at 25 °C for 15 min and added directly to the cells in 2 ml of KSFM. In general, the final plasmid concentration in all groups was adjusted to 2 $\mu$g of DNA per 4 $\mu$l of Fugene-6 reagent per 35-mm dish by addition of empty expression vector. Keratinocytes were treated with KSFM in the presence or absence of TPA or the indicated concentration of curcumin beginning 24 h after transfection. After an additional 24 h, the cells were then harvested and assayed for luciferase activity using Promega luciferase assay. All assays were performed in triplicate, and each experiment was repeated a minimum of three times. Luciferase activity was normalized per microgram of protein. Transfection efficiency was determined using a green fluorescent protein-expressing plasmid (Clontech).

*Preparation of Cell Extracts and Immunoblot Analysis*—Seventy percent confluent human keratinocytes were treated with 50 ng/ml TPA in the absence or presence of 20 $\mu$M curcumin. After 24 h, cells were rinsed with phosphate-buffered saline and lysed in cell lysis buffer (#9803, Cell Signaling), and equivalent amounts of protein were electrophoresed on acrylamide gels and transferred to nitrocellulose membrane. The membrane was blocked in 5% nonfat dry milk solution, incubated with the primary antibody, washed, and exposed to horseradish peroxidase-conjugated secondary antibody. Chemiluminescence detection reagents were used to detect the specific secondary antibody binding.

*Gel Mobility Shift and Supershift Analysis*—The gel mobility shift and gel mobility supershift assays utilizing the double-

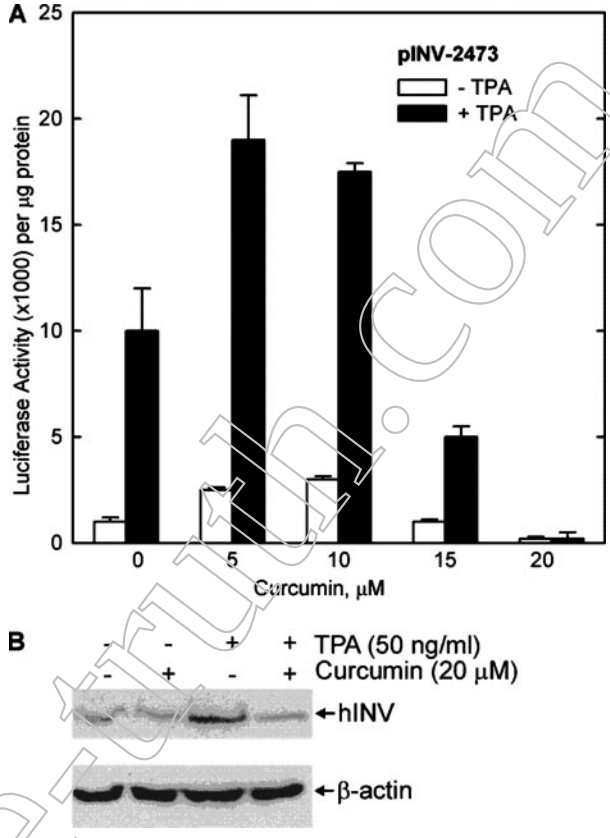


FIGURE 1. **Curcumin inhibits TPA-dependent hINV gene expression.** *A*, normal human keratinocytes growing in 9.5-cm$^2$ dishes were transfected with 2 $\mu$g of pINV-2473. After 24 h, the cells were treated in the absence (*open bars*) or presence (*solid bars*) of 50 ng TPA/ml, and the indicated concentration of curcumin. After an additional 24 h, the cells were harvested and total extracts were prepared for luciferase assay. *B*, cultured human keratinocytes (80% confluent) were treated with the indicated agents for 24 h, and total cell extracts were assayed for hINV expression by immunoblot. $\beta$-Actin was blotted as an internal protein loading control. The *error bars* indicate the mean ± S.D., and the results are representative of three independent experiments.

stranded, $^{32}$P-end-labeled oligonucleotide, 5′-TGTGGTGA-GTCAGGAAGGGGTT, and were performed as previously described (24). This sequence matches the hINV gene sequence, and the API-1 site is underlined.

*Cell Proliferation and Cell Cycle Analysis*—Keratinocytes, maintained in 35-mm dishes in KSFM, were treated for 4 days with KSFM containing TPA in the absence or presence of curcumin. Fresh medium and treatment agent were added after 2 days. The cells were then harvested with Hanks' balanced salt solution containing 0.025% trypsin and 1 mM EDTA, and the cell number was determined using a Coulter counter. For cell cycle analysis, 60% confluent cultures were treated with KSFM or KSFM supplemented with 50 ng of TPA/ml or 20 $\mu$M curcumin. After 24 h, the cells were washed with phosphate-buffered saline, fixed in methanol, and stained with propidium iodide prior to flow cytometric analysis (25).

*Fra-1 and JunB Immunoprecipitation and Immunoblot Analysis*—Subconfluent keratinocytes cultures were treated without or with 20 $\mu$M curcumin. The cells were then washed with phosphate-buffered saline and harvested in lysis buffer consisting of 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM $\beta$-glycerophosphate, 1 mM sodium orthovanadate,

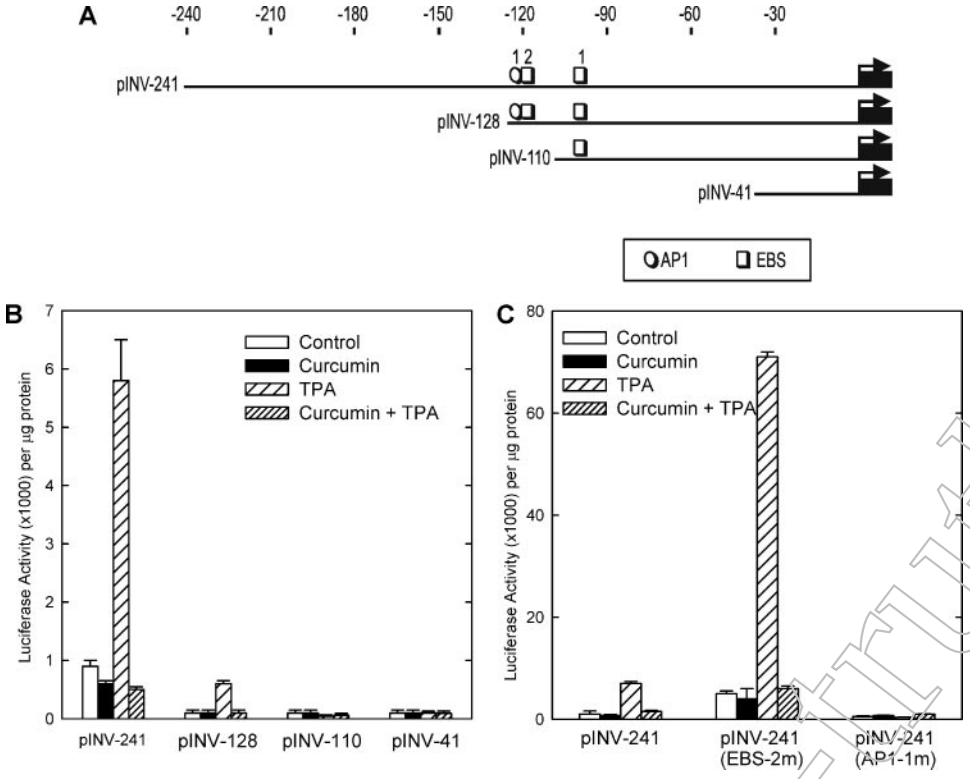


FIGURE 2. **The hINV promoter AP1-1 site is required for TPA- and curcumin-dependent gene regulation.** *A*, structure of human involucrin promoter-luciferase reporter constructs. The *narrow line* indicates hINV promoter sequence, the *black box* represents the luciferase reporter gene, and the *arrow* indicates the transcription start site and direction of transcription. The nucleotide numbering starts at position −1, adjacent to the transcription start site (56, 57). The AP1 factor binding site (AP1-1) and the ets factor binding sites (EBS-1 and EBS-2) are indicated. *B* and *C*, 70% confluent human keratinocyte cultures were transfected with 2 μg of indicated involucrin promoter reporter plasmids. The cells were then treated for 24 h with 50 ng of TPA/ml or 20 μM curcumin, and extracts were prepared for assay of luciferase activity. The AP1-1m and EBS-2m mutants sequences have been described previously (26). These data are representative of four independent experiments.

1 mg/ml leupeptin, and 2 mM phenylmethylsulfonyl fluoride. Protein lysate (100 μg of protein) was incubated with 2 μg of rabbit polyclonal Fra-1 or JunB antibody for 2 h at 4 °C. Protein A/G-agarose (25 μl) was then added to the samples followed by overnight mixing at 4 °C. The mixture was then centrifuged, and the pellet was washed three times with lysis buffer. The pellet was then resuspended in 2× Laemmli sample buffer, boiled, and electrophoresed on a 10% polyacrylamide gel for immunoblot analysis using a mouse monoclonal anti-ubiquitin antibody (Santa Cruz Biotechnology, SC-8017).

*Proteasome Activity Assay*—Subconfluent keratinocyte cultures, maintained in 35-mm dishes, were treated with 20 μM curcumin prior to preparation of total cell extracts according the manufacturer's instructions (Proteasome Activity kit APT280, Chemicon International). Briefly, 50 μg of cell lysate was incubated in assay buffer containing 25 mM HEPES, pH 7.4, 0.05 mM EDTA, 0.05% Nonidet P-40, 0.001% SDS, and 25 μg of the proteasome substrate, LLVY-AMC, for 90 min at 37 °C. Enzyme activity was measured by monitoring release of the fluorescent LLVY-AMC cleavage product, 7-amino-4-methylcoumarin.

## RESULTS

*Curcumin Is a Potent Inhibitor of TPA-induced hINV Gene Expression*—We first examined the impact of curcumin treatment on the TPA-dependent increase in hINV gene promoter activity using pINV-2473, a plasmid that encodes the full length of hINV upstream regulatory region and TATA box linked to the luciferase reporter gene (24). Keratinocytes were transfected with pINV-2473 and then treated for 24 h with TPA and/or curcumin. As shown in Fig. 1*A*, an 8-fold increase in pINV-2473 activity was observed in the TPA-treated cells. Incubation of TPA-treated cells with increasing concentrations of curcumin resulted in a curcumin concentration-dependent reduction in promoter activity. Basal and TPA-responsive promoter activity was increased at intermediate curcumin concentrations, but activity was inhibited at curcumin concentrations ≥15 μM. We next monitored the impact of curcumin treatment on endogenous hINV levels. Fig. 1*B* shows that TPA treatment increased hINV levels by 2.5-fold; however, addition of 20 μM curcumin completely suppressed this response. These findings suggest that curcumin influences endogenous hINV levels by regulating hINV promoter activity.

*Curcumin Action Is Mediated via an AP1 Factor Binding Site*—To identify the DNA binding site responsible for curcumin action we examined a series of hINV promoter truncation mutants. The proximal regulatory region (nucleotides −241/−7) of the hINV gene promoter is known to encode key regulatory elements (24) (Fig. 2*A*). As shown in Fig. 2*B*, curcumin treatment suppressed the TPA-dependent increase in pINV-241 and pINV-128 activity. However, neither TPA nor curcumin regulated the activity of constructs pINV-110 and pINV-41, indicating that the key regulatory region is located in the −128/−110 segment, which encodes the AP1-1 site (Fig. 2*A*). To identify the actual regulatory site, we monitored the impact of curcumin treatment on activity of constructs encoding point mutations at the AP1 and EBS-2 sites. As shown in Fig. 2*B*, TPA treatment increased pINV-241 and pINV-241(EBS-2m) activity, and this response was suppressed by curcumin treatment. We have previously shown that the EBS-2 site functions as a general repressor of transcription (26); therefore, it is not surprising that mutation of this site increased overall promoter activity but did not alter the curcumin-associated response. In contrast, mutation of the AP1-1 site resulted in a loss of response, suggesting that the AP1-1 site is required for this regulation.

*Curcumin Inhibits Novel PKC-dependent hINV Promoter Activity*—TPA activates hINV gene expression via a signaling cascade that includes novel PKC, Ras, MEKK1, and MEK3, which regulates activity of the p38δ-ERK1/2 complex (26–28).

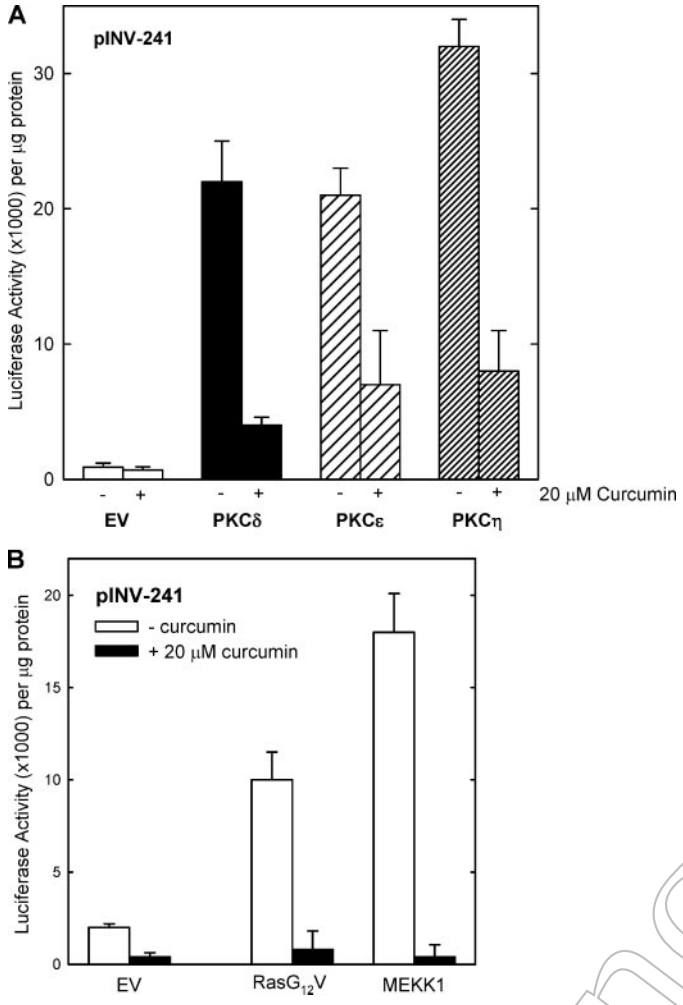


FIGURE 3. **Curcumin inhibits novel PKC-, constitutively active Ras-, and MEKK1-dependent hINV promoter activation.** *A*, near-confluent keratinocyte cultures were transfected with the 1 $\mu$g of pINV-241 reporter plasmid in the presence of either 1 $\mu$g of empty expression vector (*EV*) or plasmids encoding PKC$\delta$, PKC$\epsilon$, or PKC$\eta$. After 24 h, the cells were treated with medium containing 20 $\mu$M curcumin. After an additional 24 h, cell lysates were prepared for measurement of luciferase activity. The values are the mean ± S.E., $n = 4$. *B*, curcumin suppresses constitutively active Ras- and wild-type MEKK1-dependent hINV promoter activity. Keratinocytes were transfected as above with 1 $\mu$g of pINV-241 and 1 $\mu$g of the indicated expression vector. Empty expression vector (*EV*) was transfected in control groups. At 24 h post-transfection, the cells were treated with 20 $\mu$M curcumin for 24 h. The cells were harvested, and extracts were prepared for luciferase activity assay. The *error bars* indicate the mean ± S.E., $n = 3$.

To understand the mechanism of curcumin action, we tested the ability of curcumin to inhibit pINV-241 promoter activation induced by overexpression of the novel PKC isoforms (PKC$\delta$, -$\epsilon$, and -$\eta$), constitutively active Ras, and wild-type MEKK1. As shown in Fig. 3*A*, expression of the novel PKC isoforms caused an increase in promoter activity that was inhibited by curcumin treatment. In addition, as shown in Fig. 3*B*, promoter activation, following treatment with constitutively active Ras ($RasG_{12}V$) or wild-type MEKK1, was also inhibited by treatment with curcumin.

*Curcumin Inhibits TPA-dependent AP1 Factor Function*—The above experiments, using the hINV promoter mutants, suggest that the AP1-1 site, which is located in the proximal regulatory region of hINV upstream regulatory region, is required for curcumin-dependent regulation. To assess the impact of curcumin treatment on AP1 factor function, we determined whether curcumin influences AP1 factor level and/or AP1 factor binding to the AP1-1 site. Nuclear extracts, prepared from untreated and TPA- or curcumin-treated keratinocytes, were incubated with trace amounts of $^{32}$P-labeled AP1-1 site-encoding oligonucleotide ([$^{32}$P]AP1-1). It is important to note that the composition of AP1 factors bound at this site does not change in control *versus* TPA-treated cells (24). As shown in Fig. 4*A*, in the absence of nuclear extract, the free probe migrated at the gel front (*FP*). Addition of nuclear extract resulted in reduced migration, indicative of [$^{32}$P]AP1-1 interaction with nuclear proteins. Extracts prepared from untreated and curcumin-treated cultures displayed a similar level of binding to [$^{32}$P]AP1-1. However, there was a substantial increase in protein-associated [$^{32}$P]AP1-1 in extracts prepared from TPA-treated cells. It is of considerable interest that this level was decreased to basal levels in TPA/curcumin-treated cells. Fig. 4*B* shows that the protein interaction was specific, because binding was competed by non-radioactive AP1-1, but not by the DNA sequence encoding an Sp1 transcription factor binding site (Sp1c). Previous studies indicate that JunB and Fra-1 interact at the AP1-1 site (24). To assess the impact of curcumin treatment on this interaction, we performed gel mobility supershift analysis. Fig. 4*C* confirms that JunB and Fra-1 interacted at the AP1-1 site in TPA-treated cells and that treatment with curcumin plus TPA resulted in a substantial reduction in this interaction. The reduction in Fra-1/JunB-[$^{32}$P]AP1-1 interaction, in the presence of curcumin, could be caused by a reduction in AP1 factor expression. To assess this possibility, we prepared total nuclear extracts and monitored the level of each of the AP1 family members by immunoblot. This analysis revealed a marked increase in c-Jun, junB, junD, Fra-1, Fra-2, and fosB levels in the TPA-treated cells and a suppression of this increase in the presence of curcumin (Fig. 5*A*).

*The Role of Proteasome Activity*—We previously reported that curcumin-dependent suppression of C/EBP$\alpha$ and C/EBP$\beta$ levels in human keratinocytes requires proteasome function (18). To assess whether the curcumin-associated reduction in AP1 factor level requires proteasome function, cells were treated with curcumin or TPA in the presence or absence of MG132, a proteasome inhibitor. Our results clearly demonstrate that the curcumin-dependent reduction in Fra-1 and JunB level is reversed by treatment with MG132, indicating that the curcumin-dependent response requires proteasome activity (Fig. 5*B*).

To determine whether Fra-1 and JunB are targeted for proteasome-dependent degradation following curcumin treatment, total cell extracts from curcumin-treated keratinocytes were immunoprecipitated with anti-Fra-1 or anti-JunB. The precipitated complex was then electrophoresed for immunoblot detection of ubiquitin. As shown in Fig. 6*A*, curcumin treatment caused a substantial increase in the level of ubiquitinated Fra-1 and JunB. This increase is particularly evident at 16 and 24 h following curcumin treatment. To compare this increase to the overall change in ubiquitination, the level of ubiquitinated protein was monitored in total cell extracts. As shown in Fig. 6*B*, an increase in ubiquitination was observed as

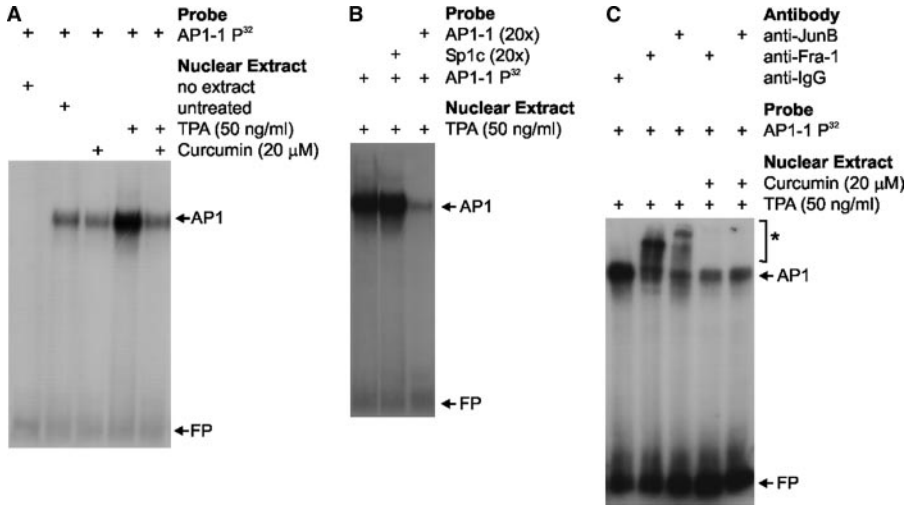


FIGURE 4. **Curcumin inhibits AP1 factor binding to hINV AP1-1 site.** *A*, keratinocytes were treated with 50 ng of TPA/ml in the absence or presence of 20 $\mu$M curcumin. After 24 h, nuclear extracts were prepared and incubated with [$^{32}$P]AP1-1 followed by electrophoresis on a 6% non-denaturing acrylamide gel. AP1 (*arrow*) indicates migration of the gel-shifted protein-[$^{32}$P]AP1-1 complex (24). FP indicates migration of non-complexed free probe. *B*, AP1 factor binding is specific. Nuclear extract, prepared from keratinocyte following treatment with TPA, was incubated with [$^{32}$P]AP1-1 without or with a 20-fold molar excess of Sp1c or AP1-1 oligonucleotides followed by non-denaturing acrylamide gel electrophoresis. Migration of the AP1 complex and free probe are indicated. *C*, Fra-1 and JunB are present in the complex. Nuclear extract prepared from TPA-treated human keratinocytes were incubated with [$^{32}$P]AP1-1 in the absence or presence of Fra-1 or JunB-specific antibodies. AP1 (*arrow*) indicates migration of the gel-shifted AP1 factor-DNA complex, and the *asterisk* indicates migration of supershifted AP1-DNA-antibody complexes. Similar results were observed in each of three separate experiments.

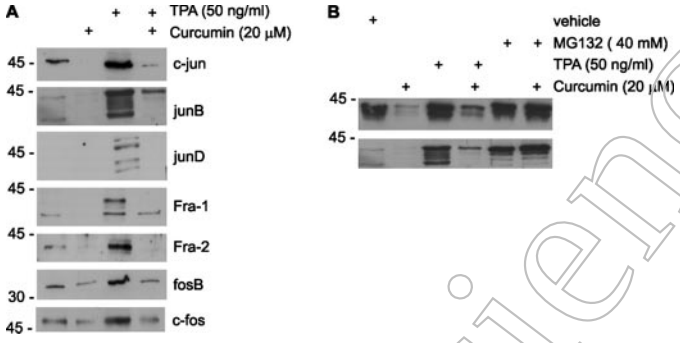


FIGURE 5. **Curcumin suppresses the TPA-dependent increase in AP1 factor levels.** *A*, keratinocytes were treated with the indicated agents for 24 h prior to preparation of nuclear extracts. The nuclear extracts were electrophoresed on a 10% denaturing acrylamide gel and then incubated with specific AP1 factor-specific antibodies and secondary antibodies. Chemiluminescent detection reagents were used to detect secondary antibody binding. *B*, inhibition of proteasome function reduces the curcumin-dependent suppression of Fra-1 and Jun B levels. Near-confluent human epidermal keratinocytes were treated for 24 h with KSFM (control) or KSFM supplemented with 20 $\mu$M curcumin in the presence or absence of 40 $\mu$M MG132. Nuclear extracts were then prepared for detection of Fra-1 and JunB levels by immunoblot. Similar results were observed in each of four independent experiments.

early as 2 h after initiation of curcumin treatment. However, the increase was modest compared with the increase in JunB and Fra-1 ubiquitination. As shown in Fig. 6*C*, the increase in Fra-1 and JunB ubiquitination occurred in parallel with a reduction in Fra-1 and JunB protein levels. We also monitored proteasome activity using a synthetic proteasome substrate, LLVY-AMC. We observed a 15 $\pm$ 3% ($n$ = 3) increase in proteasome after 2 h of treatment with 20 $\mu$M curcumin. This increase in activity was maintained between 2 and 24 h. Thus, these data correlate nicely with the ubiquitination data shown in Fig. 6*B*.

*Curcumin Treatment Impacts Cell Cycle Regulatory Proteins*—We next monitored the impact of curcumin treatment on keratinocyte cell number. As shown in Fig. 7*A*, curcumin produces a concentration-dependent reduction in cell number. During a growth period of 4 days, cell number increased 3-fold in untreated cells; however, curcumin treatment resulted in a reduction in this increase that is optimal at curcumin concentrations of >15 $\mu$M. We should note that treatment with 50 ng/ml TPA results in maintenance of cell number at the seeding density and that co-treatment with 50 ng/ml TPA and increasing levels of curcumin produces a concentration-dependent reduction in cell number at curcumin concentrations >10 $\mu$M. The curcumin-dependent reduction was associated with morphological changes that include a retraction of the cells away from each other, the formation of spindle-like processes, and less adherence to the substratum (Fig. 7*B*).

The mechanism of reduction in cell number could involve altered expression of cell cycle regulatory proteins, or activation of cell death processes. To assess this, we examined the impact of curcumin treatment on cell cycle regulatory protein level. Consistent with the idea that curcumin may inhibit cell cycle progression, curcumin treatment reduced the levels of cyclin A, cyclin B1, cyclin D1, and cyclin E (Fig. 7*C*). Among the cyclin-dependent kinases, cdk1 level was markedly reduced, but the levels of cdk2, cdk4, and cdk6 were only slightly altered. We expected that curcumin might increase cyclin-dependent kinase inhibitor levels. However, p27 level was not altered, and we paradoxically observed that curcumin treatment reduced the levels of p16 and p21. It is important to note that the overall composition of AP1 factors interacting at the AP1-1 site did not change in control *versus* TPA-treated cultures (24) (not shown).

*Curcumin Activates Apoptosis*—We next assessed the impact of curcumin treatment on apoptosis. In keratinocytes, apoptosis is associated with alteration of the Bax/Bcl-xL ratio, caspase activation, and poly(ADP-ribose) polymerase cleavage (29, 30). As shown in Fig. 8*A*, curcumin treatment reduced Bcl-xL level but did not regulate Bax level. In contrast, TPA did not influence Bcl-xL or Bax levels. Co-treatment of keratinocytes with curcumin and TPA resulted in suppression of Bcl-xL level, indicating that the curcumin response is dominant. To assess the impact on mitochondria, we treated the cells for 24 h with curcumin, TPA, or a combination of both and then stained with MitoSensor reagent, a dye that fluoresces red when aggregated in healthy mitochondria and green when released from dysfunctional mitochondria. As shown in Fig. 8*B*, loss of mito-

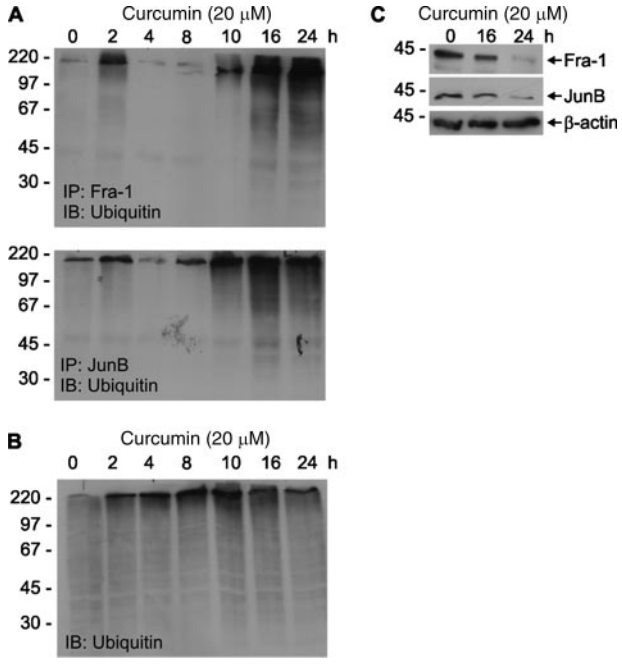


FIGURE 6. **Curcumin impact on AP1 factor ubiquitination.** *A*, curcumin treatment increases Fra-1 and JunB ubiquitination. Keratinocytes were incubated in the presence of 20 μM curcumin for the indicated times followed by the preparation of total cell extracts and precipitation with anti-Fra-1 or anti-JunB. The precipitate was then electrophoresed on a 10% polyacrylamide gel for immunoblot with anti-ubiquitin. *B*, curcumin impact on general ubiquitination. Total cell extracts, prepared after curcumin treatment as outlined above, were electrophoresed, and the blots were incubated with anti-ubiquitin. *C*, curcumin treatment reduces Fra-1 and JunB levels. Keratinocytes were treated without or with 20 μM curcumin for 16 and 24 h. Total cell extracts were then prepared for incubation with anti-Fra-1, anti-JunB, and anti-β-actin.

chondrial potential was observed only in cells treated with curcumin. These findings suggest that curcumin enhances keratinocyte apoptosis, which is generally associated with an accumulation of cells having sub-G1 DNA content. To monitor this, cells were treated for 24 h with TPA or curcumin and then harvested for cell cycle analysis. Indeed, as shown in Fig. 8*C*, TPA treatment did not markedly increase the number of sub-$G_1$ events; however, curcumin treatment resulted in a substantial increase. Cytochrome *c* release and formation of the apoptosome ultimately leads to caspase cleavage (31, 32). To assess the impact of curcumin treatment on these events, total cell extracts were prepared from curcumin or TPA-treated cells, and the procaspase level was monitored by immunoblotting. As shown in Fig. 9, reduced level of procaspases 9, 8, and 3, and poly(ADP-ribose) polymerase were observed in curcumin-treated cells.

## DISCUSSION

*Curcumin Suppresses AP1 Factor Level and AP1 Factor Binding to the hINV Promoter AP1-1 Site*—Curcumin is the active polyphenol constituent of turmeric, a common spice and food-coloring agent. Published studies suggest that curcumin is an effective inhibitor of tumor cell proliferation and tumor growth (3–9). However, the effect of curcumin on normal keratinocyte cell function has not been adequately studied. Studying the impact of curcumin on keratinocyte function is important, because keratinocytes are the major cell type present in the epidermis and, therefore, are the primary carcinogen targets.

To study the impact of curcumin on keratinocyte differentiation, we selected a gene, involucrin, which is specifically expressed in the differentiated suprabasal epidermal layers (20). Involucrin expression is increased via activation of a novel PKC, Ras, MEKK1, and MEK3 cascade that alters activity in the p38δ-ERK1/2 complex (26, 27, 33). Activation of p38δ and reduction of ERK1/2 activity leads to increased levels of C/EBP and AP1 transcription factors. These factors, in turn, bind to hINV gene regulatory elements to increase expression (26, 33). TPA is a keratinocyte-differentiating agent that increases hINV gene expression by activating this cascade (24). Our present study shows that curcumin inhibits the TPA-dependent increase in hINV promoter activity and the TPA-dependent increase in endogenous hINV expression. Additional studies demonstrate that curcumin inhibits promoter activation following overexpression of the novel PKC isoforms (δ, ϵ, and η), constitutively active Ras, and wild-type MEKK1. These findings suggest that curcumin may interfere with steps in the cascade downstream of MEKK1 activation.

This cascade targets AP1 factors (22, 24, 26). The AP1 transcription factor family consists of Jun and Fos members (34). These proteins form Jun factor homodimers and Jun/Fos factor heterodimers that bind to the AP1 factor DNA consensus sequence to regulate gene expression (34, 35). The hINV promoter AP1-1 site, located in the proximal regulatory region, binds a selected subset of AP1 factors that include JunB and Fra-1 (24). Our present studies show a curcumin-dependent reduction in binding of AP1 factors to this site, suggesting that curcumin may act to reduce promoter activity by directly interfering with AP1 factor-DNA interaction. In fact, previous studies suggest that curcumin does influence AP1 transcription factor interaction with DNA. *In vitro* treatment of recombinant AP1 proteins with curcumin results in reduced binding to the AP1 factor complex to DNA (36–38). However, this effect is not likely to explain our present findings, because we observe a curcumin-dependent reduction in promoter activity at concentrations in the range of 10–20 μM, whereas the $IC_{50}$ for inhibition of AP1 factor interaction with DNA *in vitro* is 480 μM (36–38). A second possibility is that the reduction in promoter activity is causes by the curcumin-dependent inhibition of co-activator-associated acetyltransferase activity. Our unpublished studies indicate that p300 serves as a cofactor with Fra-1 to increase hINV promoter activity.[3] Whereas this is a formal possibility, we believe that a third explanation is most likely, that the response is due to a curcumin-dependent reduction in intracellular AP1 factor levels. This explanation is based on immunoblot evidence showing that curcumin treatment reduces nuclear JunB and Fra-1 levels in keratinocytes. JunB and Fra-1 are known to associate with the hINV promoter AP1-1 site (24). These findings are consistent with the idea that curcumin-dependent regulation of AP1 factor levels is important for the response.

Curcumin has been shown to influence the AP1 factor level in other cell types. Treatment of murine keratinocyte JB6 cells

[3] J. F. Crish and R. L. Eckert, unpublished observation.

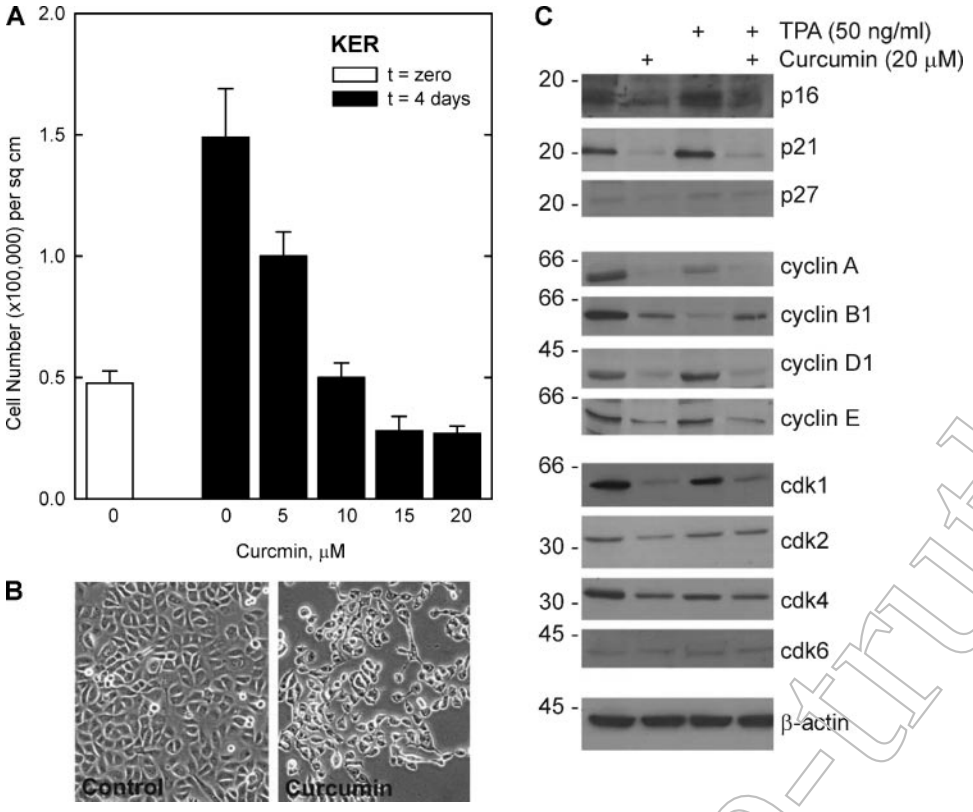


FIGURE 7. **Curcumin reduces keratinocyte cell number.** *A*, keratinocytes (10,000 cells/cm$^2$) were plated in 9.5-cm$^2$ dishes and allowed to attach overnight. The cells were then treated with the indicated concentration of curcumin, and the cell number was determined after 4 days of treatment. The *open bar* indicates cell number on day zero, and the *shaded bars* indicate cell number after 4 days of treatment (mean ± S.E., $n = 3$). *B*, curcumin alters keratinocyte cell morphology. Keratinocytes were photographed at 24 h after initiation of treatment with 20 μM curcumin. *C*, curcumin modulates cell cycle regulatory protein expression. Normal human keratinocytes were treated with the indicated agents. After 24 h, cell lysates were prepared for detection of cell cycle regulatory proteins by immunoblot. β-Actin level was determined to assure equal protein loading. Similar results were observed in three different experiments.

with curcumin results in a reduction in c-*jun* mRNA (36), and in HeLa cells curcumin treatment produces an increase in Fra-1 level and a reduction in c-*fos* level (10). Curcumin treatment of rheumatoid synovial cells reduces c-*fos* level (39). Curcumin also inhibits the TPA-dependent increase in c-*jun* mRNA and protein level in HT-1080 human fibrosarcoma cells (38). Our studies are unique in that curcumin treatment of human keratinocytes appears to produce a global reduction in the level of all AP1 factors, including c-*jun*, junB, junD, Fra-1, Fra-2, and fosB. This suggests that curcumin treatment is likely to influence a wide range of AP1 factor-dependent endpoints in keratinocytes.

*Curcumin and Proteasome Function*—Our present studies demonstrate that treatment with MG132, a proteasome inhibitor, inhibits the ability of curcumin to reduce AP1 factor levels, suggesting that proteasome function is required for curcumin action. This finding is consistent with previous reports indicating that control of AP1 factor level involves proteasome function (40, 41). It is interesting that curcumin action requires proteasome function, because curcumin has been reported to variably influence the proteasome. In neurites, curcumin treatment inhibits proteasome activity and enhances neurite outgrowth (42, 43). In skeletal muscle, proteasome function is also attenuated by curcumin (44). In mouse neuro 2a cells, curcumin suppresses proteasome function, which subsequently leads to apoptosis (45). In contrast, in HeLa cells, curcumin treatment enhances proteasome-dependent degradation of Id3 (46). Our studies suggest that the reduction in AP1 factor expression in keratinocytes requires proteasome function. This finding is consistent with a previous report indicating that the curcumin-dependent reduction in C/EBP factor level in keratinocytes requires proteasome function (18). Proteins that are proteasome substrates are polyubiquitinated as an initial step in targeting to the proteasome. Our studies indicate that ubiquitination of Fra-1 and JunB is markedly increased in curcumin-treated cells. Taken together, the findings suggest that in epithelial cells, including epidermal keratinocytes, proteasome function is required for curcumin action.

*Curcumin Modulates Cell Cycle Regulatory Protein Expression*—Curcumin has been reported to inhibit cell proliferation, induce apoptosis, and promote accumulation of cells in the $G_2$/M phase of the cell cycle (47). Our data indicate that curcumin treatment causes a reduction in keratinocyte cell number and that this is associated with an increased number of sub-$G_1$ cells, but there is no consistent change in the number of cells in the other cell cycle phases. To assess the mechanism responsible for the reduced cell number, we monitored the impact on cell cycle regulatory protein and apoptotic marker expression. Cell cycle progression is controlled by a set of protein complexes, including the cyclin-dependent kinases (cdks) and their activation partners, the cyclins (48). Increased cyclin levels are associated with increased cdk activity and increased cell cycle progression. In contrast, the cdk inhibitory proteins (p21, p27, and p16) inhibit cdk activity.

Inhibition of breast, prostate, and squamous cell carcinoma cell proliferation by curcumin is associated with reduced cyclin D1 expression via a mechanism that involves both transcriptional and post-transcriptional mechanisms (49). A similar reduction in cyclin D1 level is observed in curcumin-treated human lung epithelial cells and mammary gland epithelial cells (50, 51). Our studies are consistent with these findings, in that cyclin D1 levels are reduced. However, we also observe a reduction in cyclin A, cyclin B1, and cyclin E expression, suggesting that in keratinocytes the response is more global and that curcumin impacts multiple phases of the cell cycle. In the PC3 human prostate cancer cell line, curcumin treatment results in an increase in the level of the p21, the cyclin-dependent kinase

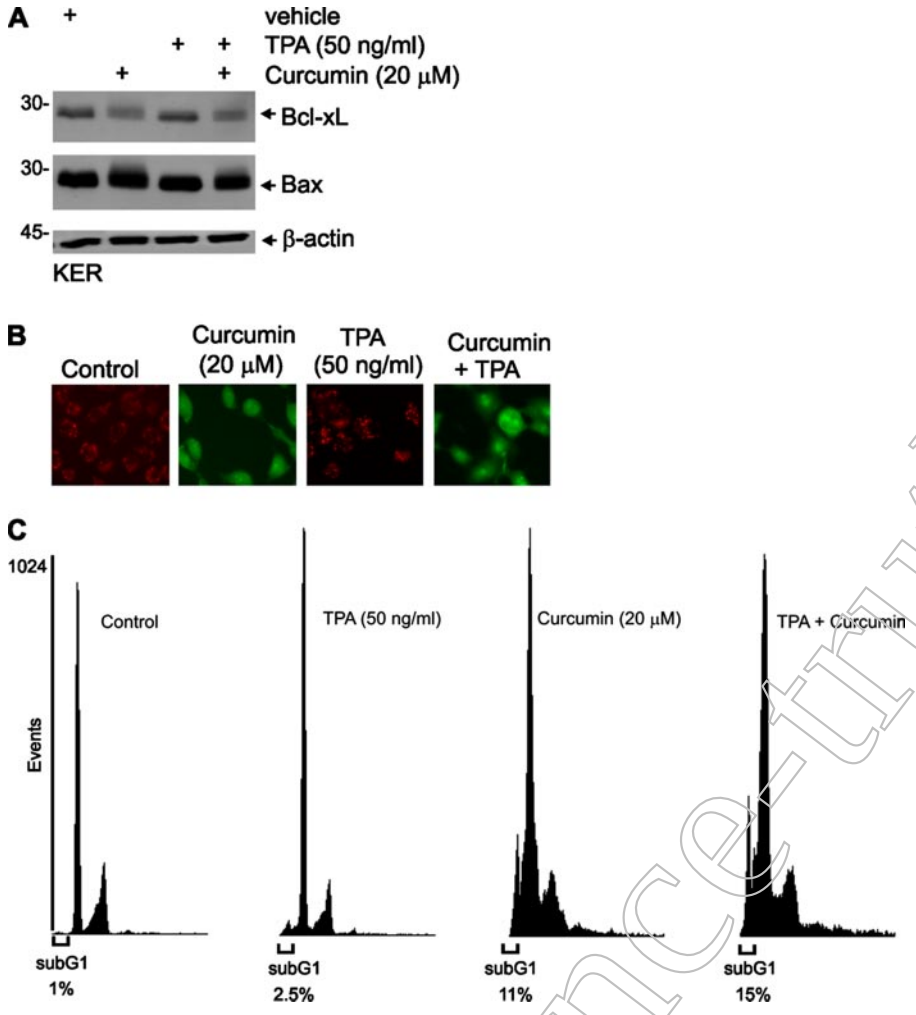


FIGURE 8. **Curcumin activates apoptosis in human keratinocytes.** *A*, curcumin treatment reduces Bcl-xL level. Near-confluent cultures were treated for 24 h with the indicated agents. Total cell extracts were prepared and electrophoresed for immunoblot with antibodies specific for Bax, Bcl-xL, and β-actin. *B*, keratinocytes, treated as indicated above, were stained with MitoSensor dye, and distribution of the dye was monitored by epifluorescence microscopy. *C*, keratinocytes, treated as above, were trypsinized, washed, fixed in methanol, and stained with propidium iodide prior to cell cycle analysis. The sub-$G_1$ DNA content events are indicated as a percentage of the total. Similar results were observed in each of three experiments.

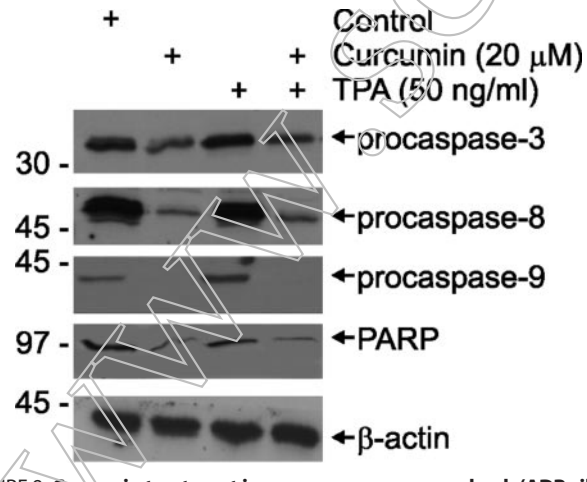


FIGURE 9. **Curcumin treatment increases procaspase and poly(ADP-ribose) polymerase cleavage.** Keratinocytes were treated with the indicated concentration of curcumin or TPA for 24 h. Total extracts were then prepared and assayed for the level of the indicated proteins by immunoblot. Similar results were observed in each of four separate experiments.

inhibitor (52). A surprising finding in our study is the curcumin-dependent suppression of cyclin-dependent kinase inhibitor levels. The levels of p16, p21, and p27 are all reduced. This finding is contrary to expectation, because a reduction of the level of these regulators is normally associated with enhanced cell proliferation. We cannot explain this finding in the context of the curcumin-dependent inhibition of cell proliferation, but we speculate that the reduction in cdk inhibitor levels may be a result of the ongoing apoptotic process. However, we are not surprised, considering that cyclin levels are reduced, that the cells cease proliferation.

*Impact of Curcumin Treatment on Keratinocyte Apoptosis*—We next determined whether the reduction in keratinocyte cell number may be due to enhanced apoptosis. Apoptosis is a type of programmed cell death that involves chromatin condensation, cell shrinkage, and nuclear and cytoplasmic destruction (53, 54). This process is associated with activation of caspases, which are cysteine proteases that are responsible for the destruction of intracellular constituents (31). In keratinocytes, this process is triggered by a shift in the ratio of Bcl-xL to Bax due to either a reduction in Bcl-xL level or an increase in Bax expression (29, 30). Our studies indicate curcumin treatment results in reduced Bcl-xL expression. This is associated with loss of mitochondrial membrane potential, reduced procaspase levels, and accumulation of cells having sub-$G_1$ DNA content. These findings strongly suggest that the keratinocytes are undergoing apoptosis. Previous studies indicate that curcumin causes apoptosis in DU145 and LNCaP prostate cancer cells that is associated with reduced Bcl-2 and Bcl-xL levels and procaspase 3 and procaspase 8 activation (49, 55). We propose that the reduction in the Bcl-xL prosurvival protein results in a shift in the Bcl-xL/Bax ratio in favor of Bax and that this leads to the release of cytochrome *c*, apoptosome formation, caspase cleavage, and loss of mitochondrial membrane potential.

*Normal Human Keratinocytes, Mechanism of Chemopreventive Agent Action*—Our present studies indicate that curcumin suppresses keratinocyte differentiation and proliferation and enhances keratinocyte apoptosis. We have previously monitored the response of keratinocytes to treatment with other chemopreventive agents. In response to treatment with green tea polyphenol, (−)-epigallocatechin-3-gallate, keratinocytes cease proliferation and differentiate (18, 22). In contrast, apige-

nin, an anti-cancer plant polyphenol derived from alfalfa sprouts, suppresses keratinocyte proliferation and differentiation. The present studies indicate that curcumin treatment suppresses keratinocyte proliferation and differentiation and enhances apoptosis. These findings point to the very interesting conclusion that these various chemopreventive agents produce beneficial actions via different general mechanisms. All three agents suppress proliferation. However, only cdk inhibition promotes differentiation, and only curcumin promotes apoptosis. These findings suggest that these agents may have complimentary actions for the treatment of surface epithelial cell disease and that this should be considered when designing therapies. An additional important point is that the present studies examine the impact of curcumin treatment on normal human keratinocyte survival; however, it will be important in future studies to repeat these experiments using UV light-exposed keratinocytes. UVB is an important skin cancer-promoting entity, and it will be important to determine whether curcumin produces similar changes in UV-exposed cells.

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# Curcumin Suppresses AP1 Transcription Factor-dependent Differentiation and Activates Apoptosis in Human Epidermal Keratinocytes

Sivaprakasam Balasubramanian and Richard L. Eckert

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